METHOD OF INACTIVATING RIBONUCLEASES AT HIGH TEMPERATURE

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CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of co-pending application Serial No. 10/403,395, filed March 31, 2003, the entire content of which is incorporated herein.

FIELD OF THE INVENTION

The present invention is directed to methods for protecting ribonucleic acids (RNA) from degradation by ribonucleases (RNases). Specifically, the invention includes methods for protecting RNA from RNases during storage of the RNA, as well as methods for protecting RNA from RNases present in reagents used in scientific protocols that utilize RNA (such as reverse transcriptase-polymerase chain reactions, RT-PCR). The invention further includes methods to increase the sensitivity of RT-PCR.

DESCRIPTION OF PRIOR ART

Ribonucleic acid (RNA) is an extremely important component of most biological systems. Its biologic roles include messenger RNA (mRNA), which carries the genetic code from the nucleus; ribosomal RNA (rRNA), which helps to translate the nucleic acid message to a polypeptide; and transfer RNA (tRNA), which functions to help decode messenger RNA. Further, RNAs are beginning to be recognized for a host of other regulatory functions, such as small interfering RNA and regulatory ribozymes, which have an enzymatic function. In some viruses, RNA carries the core genetic message itself.

Because of its importance in biological actions, RNA production and degradation are heavily regulated *in vivo*. While DNA is quite stable, an effect of its being a double-stranded

molecule, RNA (a single-stranded molecule) is extremely susceptible to enzymatic degradation. Enzymatic degradation is carried out by a ubiquitous class of enzymes called ribonucleases (RNases).

RNases are extremely robust enzymes. Unlike most proteins, RNases are very difficult to degrade either by extreme pH or high temperature. There are several theories as to why RNases evolved to be so robust. They include protection from the consequences of translating degenerate RNA into proteins and regulation of intracellular RNA. In addition, although RNases can be temporarily denatured by high temperatures, some RNases renature upon cooling (a phenomenon called reversible thermal denaturation) so that denaturing RNases via high temperature alone is not an effective method for protecting RNA from RNases at, say, room temperature.

RNA is an extremely important tool in molecular biology. Due to the presence of introns in eukaryotic genomic DNA, the genetic message carried in genomic DNA is not directly translatable into proteins. Therefore, genomic DNA is a second choice when making libraries, cloning, and introducing genes into a cell on a plasmid or vector. The most desirable source for libraries is complementary DNA (cDNA). cDNA is made directly from mRNA which has been back-transcribed into DNA. This process requires isolation of mRNA which has gone through the process of intron removal, a process commonly referred to as "splicing." During splicing, the non-translated introns are removed before the RNA is translated into protein. By using reverse transcriptase in the presence of deoxynucleotide bases (including thymine, instead of the uracil found in RNA), a single-stranded DNA, complementary to the mRNA, can be synthesized.

Further replication of the single-stranded DNA transcript using DNA polymerase produces a double-stranded cDNA molecule having the sequence of the mRNA template. In addition, cDNA, like genomic DNA, is very stable; thus, its utility for molecular biological manipulations is magnified. The cDNA can be used for a variety of purposes, including amplification using PCR and the creation of cDNA libraries for use in cloning. By

synthesizing cDNA, scientists have been able to create synthetic genes which, when transfected into an organism, can be directly translated into a functional protein. This capability would be impossible using the genomic DNA of a eukaryote because of the presence of introns. The introns must be properly spliced from the genomic sequence in order for a proper protein to result.

The synthesis of cDNA is not the only experimental use for RNA. Other uses, such as RNA vectors (see, for example, Zhang et al. (1997) *Virology* 233:327-338) and RNA probes, are also adversely affected by RNases. Therefore, one important research effort of the last few years has been the development of methods to protect RNA from RNases. In short, because the need to preserve RNA for analysis has been known for some time, a number of different approaches have been used for inhibiting RNase activity. The RNase activity to be eliminated from the sample may be present either through co-purification of the RNase with the RNA, or may have been introduced into the sample from reagents used in processing the sample.

Several methods for inhibiting RNase activity have been developed. These methods include the use of diethylpyrocarbonate (DEPC), the use of RNase inhibitor proteins, and the use of ribose compounds that preferentially bind to the RNase.

One method of inhibiting RNase activity involves using the chemical agent diethylpyrocarbonate (DEPC). DEPC reacts with RNases to inactivate the enzyme. However, the use of this type of chemical entity is not always convenient or even possible. (For example, due to adverse chemical reactions, solutions of Tris and MOPS cannot be treated with DEPC.) DEPC reacts with a number of different residues in RNases, leading to deactivation of the RNase enzyme. For example, in RNase A (EC 3.2.27.5), two histidine residues (His-12 and His-119) are key to the catalytic activity of the enzyme. DEPC reacts with the His-12 residue of RNase A to yield a carbamate-type bond, thus making this residue unavailable for reaction with RNA. (See Findlay et al. (1961) *Nature* 190:781-784; and Raines (1998) *Chem Rev.* 98:1045-1066.). In other types of RNases, DEPC interferes the ϵ-

amino groups of lysine and the carboxylic groups of aspartate and glutamate, both intra- and inter-molecularly, to deactivate RNases. While treatment with DEPC is effective, its use is very laborious. DEPC is also a suspected carcinogen.

When using DEPC as protection against RNases, reagents, glassware, electrophoresis equipment, and any other labware that may come in contact with the RNA is rinsed in DEPC-treated water, then incubated at 37°C for several hours to promote RNase degradation. The treated equipment is then autoclaved for approximately 30 minutes to destroy the DEPC. In addition, RNA solutions are stored in DEPC-treated water to protect the RNA during storage. When this method of storing RNA is used, the DEPC needs to be removed from the solution before using the RNA.

RNase inhibitor proteins were first identified as a protein that inhibited pancreatic RNase. This family of RNase inhibitor proteins was identified and purified from placental extracts. (See Blackburn, P. et al. (1977) *J. Biol. Chem.* 252:5904-5910.) A gene for an RNase inhibitor was subsequently cloned from the placenta, and a recombinant RNase inhibitor protein developed. (See, for example, U.S. Patent 5,552,302, to Lewis et al.) These inhibitor proteins function mechanistically by forming a very strong 1:1 complex between the inhibitor and the RNase.

The genes encoding the human placental inhibitor, as well as those from pig and rat, have been cloned and sequenced. The three-dimensional structures for some of the members of the family have also been determined. (See Kobe & Deisenhofer (1996) "Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A," *J. Mol. Biol.* 264(5):1028-1043.) Comparisons of the properties of this family of RNase inhibitor proteins have been published. (See Blackburn et al. (1977) *J. Biol. Chem.* 252:5904-5910; Burton & Fucci (1982) *Int. J. Pept. Protein Res.* 19:372-379.) The usefulness of these inhibitor proteins in molecular biology applications has resulted in their characterization to some extent. In particular, the human placental form of the inhibitor protein has been reported: (1) to inhibit RNases of the RNase A, B and C family

of enzymes; (2) to be thermally inactivated at about 55°C in aqueous solution; and (3) to be unable to inhibit the major RNase from *E. coli* (commonly referred to as RNase I) or RNases from plant sources. (See, for example, "Expressions 9.3," a publication of Invitrogen Life Technologies (San Diego, California) that describes Invitrogen's RNaseOUT-brand inhibitor. See also Ambion, Inc.'s (Austin, Texas) product literature for Ambion's RNase Inhibitor.) When the RNase is complexed to the inhibitor, the complex does not have any RNase activity. However, as reported in the above-noted product literature, the RNase is not permanently inactivated by the inhibitor. If the inhibitor is released from the inhibitor-RNase complex, under certain conditions the freed RNase will regain its ability to degrade RNA.

The RNase inhibitor protein from human placenta—either isolated from its native source or made through recombinant means—has been available commercially for a number of years. During that time, reports have been published that the inhibitor is ineffective in preventing RNA degradation in certain molecular biology applications, such as RT-PCR. This is due, reportedly, to the poor thermostability of the inhibitor protein at the temperatures used in such reactions. In fact, these publications suggest that adding the RNase inhibitor would be detrimental to successful completion of RT-PCR experiments. In short, the product literature suggests that the RNase inhibitor protein as supplied may already have a significant fraction of the inhibitor protein complexed to RNase. Further, this RNase would then be released in an active form upon heating of a solution containing the RNase inhibitor. The literature goes on to infer that the potentially active RNase released may destroy the RNA template in the experiments, thus leading to failure in the experiments.

Due to the difficulty of protecting RNA from RNases, there is a long-felt and unmet need for a better method to protect RNA from RNase degradation, both during storage of the RNA and during manipulations of the RNA. The method should be easy to implement and should not require the use of toxic reagents. The method should yield RNase-protected RNA that can be directly used (from one protocol to the next) without intervening and additional purification steps and without concern for the enzymatic degradation of the RNA.

SUMMARY OF THE INVENTION

The present inventors have discovered, quite surprisingly, that an RNase inhibitor protein from a mammalian source (human placenta, rat, etc., native or recombinant) can be combined with particular chemical conditions, such that the combination allows the inhibitor to be highly effective in specific, high-temperature applications, such as RT-PCR and quantitative RT-PCR. (Joe: these particular chemical reagents, i.e., DTT are no longer required - heat alone will work). In particular when heat is added to the RNA inhibitor solution combined with a sample suspected of containing RNase, this results not only in the inhibition of RNase in the reaction, but also results in the lack of release of active RNase following treatment of the solution under conditions that inactivate the RNase inhibitor. Insofar as the literature discussed previously directly indicates that RNase inhibitor solutions should not be heated under any conditions (as they will inactivate the RNase inhibitor and potentially release active RNase into the experimental solution), the present invention is in direct conflict with the conventional fashion in which placental RNase inhibitor is used.

Another unexpected and unpredictable aspect of the present invention is that when the RNase inhibitor solutions of the present invention are heated, the solutions are capable of inactivating RNases not normally inhibited by the RNase inhibitor alone or the added reagents alone. While not being limited to a specific mode of action, this increase in the range of RNases capable of being inactivated apparently is the result of a synergism between the RNase inhibitor and the added reagents or heat. The combination is greater than the sum of its parts; the combination inactivates RNases that are not inactivated by either the inhibitor or the added reagents separately. The net result is that the invention described and claimed herein results in the protection of RNA from mammalian RNases both *before* and after *heating* of the solution, and also provides protection from RNases derived from bacterial and plant sources after gently heating the solution.

Another unexpected and unpredictable aspect of the present invention is that the RNase inhibitor solutions of the present invention are capable of inactivating RNases even

when the reaction mixtures are devoid of reducing agents, such as dithiothreitol (DTT). In the prior art, dithiothreitol or reducing agents of similar functionality are deemed required reagents. The present inventors, however, have determined that such reducing agents are not absolutely required to inactivate RNases using the inhibitors described herein.

It is therefore a primary aim and object of the invention to provide a method for protecting RNA from RNase degradation. A first embodiment of the invention is thus directed to a method for protecting RNA from enzymatic degradation by RNases. The method comprises first, to a first solution containing RNA or to which RNA will subsequently be added, adding an amount of a second solution comprising an amount of an RNase inhibitor protein and a buffer that either contains, or is devoid of, reducing agents such as DTT. The amount of RNase inhibitor protein in the second solution is sufficient to protect RNA from enzymatic degradation by RNases present in the mixture. Then the mixture is heated to a temperature no less than about 50°C for a time sufficient to inhibit RNase activity present in the mixture. In an alternative embodiment, the mixture is heated to a temperature greater than 65°C.

In this fashion, RNA present in the mixture, or subsequently added to the mixture, is protected from enzymatic degradation by RNases in general, and mammalian RNases in particular. If RNA is to be subsequently added to the mixture, the mixture can be heated to at least about 90°C.

The preferred method protects RNA from enzymatic degradation by RNase A, RNase B, RNase C, and RNase I.

The buffer containing the RNase inhibitor protein can either contain reducing agents or be devoid of reducing agents, such as β-mercaptoethanol or DTT.

The RNase inhibitor protein is preferably derived from porcine, rat, human placental, or recombinant human placental sources. Such RNases inhibitors are available commercially, such as from Promega Corporation.

To gain the benefits of the present invention, the mixture need not be heated for a long time. Generally, about twenty (20) seconds at 50°C or higher is sufficient. (Temperatures of greater than 65°C may also be used.) The mixture, of course, can be heated for much longer periods of time, anywhere from minutes (if RNA is present) to hours (if RNA is to be subsequently added).

A second embodiment of the invention is drawn to a method of inactivating RNases in a first solution known to contain RNA and suspected of containing RNases. This second embodiment comprises adding to the first solution a second solution comprising an RNase inhibitor protein deposited in a buffer that either contains, or is devoid of, reducing agents, to yield a mixture, and then heating the mixture to a temperature of at least about 50°C for a time sufficient to inhibit RNase activity present in the mixture. This results in RNases present in the first solution, if any, being inactivated. It is preferred that the solution be heated anywhere from twenty (20) seconds to five (5) minutes.

A third embodiment of the invention is drawn to a method of storing RNA under conditions that protect the RNA from enzymatic degradation by RNases. The third embodiment comprising adding to a first solution containing isolated RNA or to which isolated RNA will subsequently be added, a second solution comprising an RNase inhibitor protein in a buffer that either contains, or is devoid of, reducing agents, to yield a mixture. The mixture is then heated to a temperature no less than about 70°C for a time sufficient to inhibit RNase activity present in the mixture; and then the mixture is cooled and stored in a suitable container.

Yet another embodiment of the invention is directed to a method of performing RT-PCR and quantitative RT-PCR. This fourth embodiment of the invention comprises first, prior to undergoing thermal cycling, adding to an RT-PCR reaction cocktail containing RNA (or to which RNA will subsequently be added) an amount of a solution comprising an RNase inhibitor protein in a buffer that either contains, or is devoid of, reducing agents, to yield a mixture. The amount of the solution added is sufficient to protect any RNA present in the

RT-PCR reaction cocktail from enzymatic degradation during a first round of thermocycling. Then, if RNA is absent from the mixture, adding RNA template to the mixture. An RT-PCR reaction is then conducted on the mixture, whereby RNA in the mixture is protected from enzymatic degradation by RNases present in the RT-PCR reaction cocktail and is also protected from enzymatic degradation by RNases during the first round of thermocycling and throughout the RT-PCR reaction.

A variation on this embodiment comprises adding a first solution containing an RNase inhibitor protein in a buffer to an RT-PCR reagent mixture, to yield a second solution. The second solution is then heated to at least about 50°C for a time sufficient to inhibit RNase activity present in the second solution. RNA is then added to the second solution to yield an RNA mixture. Lastly, an RT-PCR reaction is conducted on the RNA mixture, whereby the RNA in the RNA mixture is protected from enzymatic degradation by RNases present in the second solution and whereby the RNA in the mixture is further protected from RNases during the RT-PCR reaction.

A still further embodiment of the invention is directed to a method of inactivating RNase I. This embodiment of the invention comprises adding to a first solution suspected of containing RNase I, a second solution comprising an RNase inhibitor protein in a buffer that either contains, or is devoid of, reducing agents, to yield a mixture; and then heating the mixture to a temperature of at least about 70°C for a time sufficient to inhibit RNase I activity present in the mixture, whereby any RNase I present in the first solution is inactivated.

In any of the embodiments disclosed herein, the RNase inhibitor protein used in the method can be derived from porcine, rat, human placental or recombinant human placental sources.

The objects and advantages of the invention will appear more fully from the following detailed description of the preferred embodiment of the invention made in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a photograph of a gel illustrating inhibition of bovine pancreatic RNase using rat-derived RNase inhibitor protein in an RT-PCR protocol. See Example 1 for lane assignments.

Fig. 2 is a photograph of a gel illustrating protection of mRNA in quantitative RT-PCR using rat-derived RNase inhibitor protein. See Example 2 for lane assignments.

Fig. 3 is a photograph of a gel illustrating protection of mRNA in quantitative RT-PCR using human-derived RNase inhibitor protein. See Example 2 for lane assignments.

Fig. 4 is a histogram showing the results of a statistical analysis of band density for the products of the RT-PCR reactions described Example 2 and shown in the gels of Figs. 2 and 3.

Fig. 5 is a schematic showing of the results of a plate assay indicating the digestion of RNA by RNase. The assay comprises an agar plate loaded with agar mixed with RNA and a pH indicator. The plate is cored and the wells loaded with RNase and an RNase inhibitor, in treatments that are either heated or not. Digestion of RNA results in a visible digestion zone around the affected wells. See Example 3.

Fig. 6 shows the results of a plate assay to examine the effect of heating RNase on the degradation of RNA in the presence of an RNase inhibitor and different types of buffers. See Example 4.

Fig. 7 is a photograph of a gel illustrating protection of mRNA from degradation by RNase derived from wheat germ in an RT-PCR experiment. See Example 5.

Fig. 8 is a photograph of a gel illustrating protection of mRNA from degradation by RNase derived from wheat germ in an RT-PCR experiment. See Example 6.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods for protecting RNA from degradation by RNases. The invention is further directed to methods of storing RNA in an RNase activity-free stock solution.

Abbreviations and Definitions:

As used herein, the term "reducing agent" means any reducing agent, without limitation, including dithiothreitol and mercaptoethanol.

As used herein, the term "RNA" expressly denotes RNA from any source without limitation, including prokaryotic RNA, eukaryotic RNA, mitochondrial RNA, and RNA derived from transcription reactions.

As used herein, the unqualified term "RNase" expressly denotes RNase from any source without limitation, including prokaryotic and eukaryotic RNases. RNases are found in most organisms and in many organs and body fluids. Examples of RNases include (without limitation) RNases A, B, and C (mammalian, *e.g.*, bovine pancreatic), RNase 1 (*e.g.*, human pancreatic), RNase 2 (eosinophil-derived neurotoxin), RNase 3 (eosinophil-cationic protein), RNase 4, and RNase 5, as well as the bacterial RNases I, II, III, P, PH, R, D, T, BN, E, and M, among others. All share the primary activity of degrading RNA. For a more extensive discussion of RNases, see, for example, D'Allesio & Riordan "Ribonucleases: Structures and Functions," Academic Press, New York (1997); Sorrentino & Libonati (1997) "Structure-Function Relationships in Human Ribonucleases: Main Distinctive Features of the Major RNases," *FEBS Letters* 404:1-5; and Nicholson (1999) "Function, Mechanism, and Regulation of Bacterial Ribonucleases," FEMS *Microbiol. Rev.* 23:371-390.

As used herein, the terms "RNase inhibitor protein" or "RNase inhibitor" denotes a mammalian-derived protein that inhibits the activity of RNase. The preferred RNase inhibitor proteins for use in the present invention are those manufactured by Promega Corporation, Madison, Wisconsin. Promega markets RNase inhibitor proteins derived from human

placenta, both as a native protein and a recombinant version, under the federally-registered trademark "RNasin"®-brand RNase inhibitor (U.S. Trademark Registration No. 1,237,884). For additional information on the RNasin-brand RNase inhibitor, see Blackburn & Moore (1982) In: *The Enzymes*, Vol. XV, Part B; Blackburn, Wilson, & Moore, (1977) *J. Biol. Chem.* 252:5904; Lee et al. (1989) *Biochemistry* 28:219; Lee et al. (1989) *Biochemistry* **28**, 225. See also U.S. Pat. Nos. 4,966,964; 5,019,556; and 5,266,687.

Another preferred RNase inhibitor protein for use in the present invention is designated herein as "RNasin-Plus"™ RNase inhibitor. This RNase inhibitor protein is a recombinant protein derived from rat lung and produced in *E. coli*. For a description of the cloning of this protein, see Kawanomoto et al. (1992), *Biochim. Biophys. Acta* 1129: 335-338, which discusses the cDNA cloning and sequence of rat ribonuclease inhibitor isolated from a rat lung cDNA library. This protein can be purchased commercially from Promega Corporation, Madison, Wisconsin. The cloned RNA encoding this rat-derived RNase inhibitor is also available commercially from OriGene Technologies, Inc. (Rockville, Maryland).

The invention described herein is suitable for use in a variety of molecular biological protocols that use or require RNA. For an overview of a host of such protocols, see "RNA Methodologies, Second Edition," E. Farrell, Jr., editor, Academic Press, 1998.

The following primers where used in the Examples:

F-CGCCCCCTCGGAG (SEQ. ID. NO: 1): Luciferase RT-PCR reverse primer

F-GAAAGGCCCGG (SEQ. ID. NO: 2): Forward luc RT-PCR

F-GGGATCCTCTAGAGTCGCCA (SEQ. ID. NO: 3): downstream Kan RT-PCR

F-TTGGGCGTGTCTCAAAATCT (SEQ. ID. NO: 4): upstream Kan RT-PCR 2

HO-CGCCCCCTCGGAG (SEQ. ID. NO: 5): Luciferase RT-PCR reverse primer

HO-GAAAGGCCCGG (SEQ. ID. NO: 6): Forward primer luc RT-PCR

In an exemplary version of the instant invention, a first solution containing RNA is protected against degradation by RNases by adding to it a second solution containing an RNase inhibitor, such as "RNasin-Plus"™ brand RNase inhibitor (Promega), in a buffer. The buffer either contains, or is devoid of, reducing agents in general. Where a reducing agent is present in the buffer, DTT is preferred. In a particularly preferred embodiment, the buffer comprises Promega Buffer B or Promega Storage buffer, in the presence or absence of DTT. Thus, one preferred buffer comprises 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, and 50 mM NaCl, but is devoid of reducing agents. Another preferred buffer comprises 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, and 50% (v/v) glycerol, but is devoid of reducing agents.

After adding the RNase inhibitor and buffer, the solution is heated to at least about 50°C, preferably to at least about 70°C, for a time sufficient to inactivate RNases, generally from about 20 seconds to perhaps five (5) minutes or more. The time the solution is left at elevated temperatures will, to some extent, depend upon the protocol being undertaken. Inactivation of the RNases occurs essentially immediately for mammalian RNases, and the heating serves to deactivate more hardy RNases. If RNA is not yet present in the mixture, it may be heated for 10 minutes or longer at temperatures at least as high as 90°C. This treatment renders the mixture free from RNase activity both before and after the heating step. A distinct advantage of this approach is that the RNA in the solution is protected from RNases for an extended period of time without fear of reversible denaturation.

The RNase inhibitors that can be used in the invention include, without limitation, porcine RNase inhibitor, rat RNase inhibitor, human placental RNase inhibitor, and recombinant RNase inhibitor. This list is exemplary. There are several commercial suppliers of RNase inhibitor, including Promega Corporation.

Another embodiment of the invention is a method of protecting RNA from RNases during storage. In this embodiment, the solution containing RNA can be stored for long periods of time (*e.g.*, greater than 90 days) without concern for the degradation of the RNA by RNases. To protect the RNA, an RNase inhibitor, such as "RNasin" brand inhibitor, is

added to the RNA-containing solution, in the presence or absence of reducing agents. The solution is then heated to about at least 50°C for about at least twenty (20) seconds. The mixture is then placed in a suitable container and allowed to cool. After being so treated, the RNA solution can be stored for extended periods of time (*i.e.*, at least one (1) hour and often far longer, *e.g.* > 90 days) at room temperature, yet still be protected from RNases. A distinct advantage of this embodiment is that the treated RNA solution does not have to be placed in cold storage to be protected from RNases. Those of skill in the art will understand that cold storage (*e.g.*, 4°C or –20°C), will help alleviate temperature-dependent RNA degradation.

The invention also comprises a method to protect RNA during chemical and enzymatic reactions in general and, in particular, during RT-PCR-based protocols. In this embodiment of the invention, the RNA may have been isolated previously and may have already been protected from RNases by the disclosed invention. However, those of skill in the art will recognize that adding any reagent to an RNA-containing solution risks the introduction of non-inhibited RNases. In such an instance, an RNase inhibitor can be added to the reaction mixture before the first reaction step is performed. In the case of RT-PCR-based reactions, the RNase inhibitor is added prior to the first thermocycling step. The RNA is thereby protected from degradation by RNase during the thermocycling step and, surprisingly, in all subsequent thermocycles. In a particularly preferred embodiment, the RNase inhibitor and buffer are added to the reaction mixture prior to the addition of the RNA. Further, the reaction mixture may be heated prior to addition of the RNA, assuring the highest RNA protection and the highest sensitivity of the reverse transcriptase reaction.

The invention is also effective to inhibit RNases normally thought not to be inhibited by native mammalian or recombinant RNase inhibitors. A synergistic effect has been discovered in the combination of an RNase inhibitor protein, and heat, the combination yielding results that are greater than the sum of the individual steps alone. In this embodiment of the invention, RNase I which is produced by prokaryotes in general, and *E. coli* in particular, are inhibited. In this embodiment, as in the earlier embodiments, an RNase

inhibitor and a suitable buffer are added to a solution thought to contain RNase I to yield a second solution. The second solution is then heated to at least about 70°C for a time sufficient to inactivate the RNase I. The prokaryotic RNases are thus inactivated by the treatment, and RNA can be added without fear of degradation.

While the above methods for inactivating RNases and protecting RNA from degradation can be effected by heating the RNA solution or mixture at 70°C, it is a feature of the invention that a solution or mixture to which RNA is to be subsequently added can be heated for an extended period of time at temperatures of at least as high as 90°C or higher (essentially to the boiling point). Once the reaction is cooled, RNA can be added without fear of degradation by RNases. It is a further aspect of the invention that, upon addition of the RNase inhibitor protein to the RNA solution, the RNase will be inhibited from degrading the RNA in the solution. Moreover, after heating the mixture at a temperature of at least about 70°C, the RNases are inactivated and the RNA is safe from RNase degradation for an extended period of time (*i.e.*, at least an hour or more), at room temperature.

EXAMPLES

The following Examples are included solely to provide a more complete understanding of the invention disclosed and claimed herein. The Examples do not limit the invention in any fashion.

Example 1: Inactivation of RNase in Rat Liver Lysate by Promega's "RNasin-Plus"-Brand RNase Inhibitor:

The purpose of this Example is to demonstrate the protection of mRNA with "RNasin Plus" RNase inhibitor in an RT-PCR experiment wherein rat liver lysate (a source of RNase) was purposefully added to the reactions.

Materials:

Rat Liver Lysate: 0.5 mg/ml in nanopure water (Sigma Pt # L-1380 Lt # 108F8185)

Luciferase mRNA: 0.1 mg/ml in nanopure water (Promega Pt# L456A Lt # 14937403)

Luciferase mRNA: 0.01 mg/ml in nanopure water (Promega Pt# L456A Lt # 14937403)

"RNasin Plus"-brand RNase inhibitor*: 40 units/µl (Promega Pt # N261 Lt # 165682)

AccessQuick™ RT-PCR System (Promega Pt # A1703 Lt # 158304)

* "RNasin Plus"-brand rat-derived RNase inhibitor can be purchased commercially from Promega.

Experimental:

Two hundred (200) µl of both the "RNasin Plus"-brand inhibitor and the rat liver lysate were heated in separate tubes for 15 minutes at 70°C.

The following reactions were then assembled in duplicate without the addition of mRNA:

Table 1:

Rxn #	Nanopure Water	RNasin	Rat Liver Lysate
1	—	20 µl	—
2	17.5 µl	—	2.5 µl
3	—	20 µl	2.5 µl
4	—	20 µl	2.5 µl
5	—	20 µl	2.5 µl
6	—	20 µl	2.5 µl
7	—	20 µl	2.5 µl

Table 2:

Rxn #	Contents	RNasin Heated	Rat Liver Lysate Heated
1	RNasin Only	No	NA
2	Rat Liver Lysate Only	NA	no
3	RNasin + Lysate	No	no
4	RNasin + Lysate	Yes	no
5	RNasin + Lysate	No	yes
6	RNasin + Lysate	Yes	yes
7	RNasin + Lysate	Yes	yes

In Reaction Nos. 4, 5, and 6, the RNase inhibitor and the rat liver lysate were heated separately and then combined. In Reaction No. 7, the RNase inhibitor and the rat liver lysates were combined and then heated.

Reaction No. 7 was assembled using non-heat treated lysate and non-heat treated RNase inhibitor and then incubated at 70°C for 15 minutes.

One (1) µl of 0.1 mg/ml luciferase mRNA (100 ng) was added to the first set of reactions.

One (1) µl of 0.01mg/ml luciferase mRNA (10ng) was added to the second set of reactions. That is, the second set of reactions included a 10-fold reduction in the amount of mRNA template as compared to the first set of reactions.

The reactions were then incubated for 1 hour at 37°C.

During the incubation, an RT-PCR master mix was assembled on ice using components available from Promega Corp., as follows:

250 µl Access Quick 2x Master Mix
220 µl Nuclease-free Water
10 µl Luciferase Upstream primer, Promega Part No. 20247 (15 µM)
10 µl Luciferase Downstream primer Promega Part No. 20818 (15 µM)
10 µl AMV-RT (5 units/µl)

After a 1 hour incubation at 37°C (a temperature designed to challenge the reaction; 37°C being an optimum temperature for RNase activity), the reactions were moved on ice. Forty-five (45) µl of the RT-PCR master mix was dispensed into "MicroAmp"-brand strip-well tubes on ice. Five (5) µl of each reaction was then added to the master mix. The reactions were placed in a PE 9600 thermocycler (PerkinElmer Corporation, Shelton, Connecticut) and cycled as follows:

48°C	45 minutes	1 cycle
96°C	2 minutes	1 cycle
94°C	15 seconds	
65°C -> 55°C	1 minute	20 cycles
72°C	1.5 minutes	
72°C	5 minutes	1 cycle
4°C	Soak	

Fifteen (15) µl of each RT-PCR reaction was then loaded onto a 1% TBE agarose gel with ethidium bromide staining and run for 1 hour at 80V.

The results are shown in Fig. 1. The lane descriptions are as follows:

Lane Nos. 1 through 8 contain 100 ng mRNA.
Lane Nos. 11 through 18 contain 10 ng mRNA.
Lane Nos. 9 and 10 are blanks.

Lane No.

1. 200 b.p. DNA Step Ladder
2. RNasin Plus Only (–)
3. Lysate Only (–)
4. RNasin (–) + Lysate (–)
5. RNasin (+) + Lysate (–)
6. RNasin (–) + Lysate (+)
7. RNasin (+) + lysate (+) heated separately
8. RNasin (+) + lysate (+) heated together

11. 200 b.p. DNA Step Ladder
12. RNasin Plus Only (–)
13. Lysate Only (–)
14. RNasin (–) + Lysate (–)
15. RNasin (+) + Lysate (–)
16. RNasin (–) + Lysate (+)
17. RNasin (+) + lysate (+) heated separately
18. RNasin (+) + lysate (+) heated together

(-) = Non heated sample
(+) = Heated sample

The results of the gel shown in Fig. 1 are striking. In each of lanes 3, 5, 7, 13, 15, and 17, there is a complete lack of RT-PCR product. In contrast, in each of lanes 2, 4, 6, 8, 12, 14, 16, and 18, there is a very distinct product detected. These results indicate a distinct synergy between the inhibitor, the RNase, and heating. In particular, as shown in lanes 7 and 17, when the inhibitor and the lysates are heated separately, and then combined, there is a total failure of RT-PCR. But, as evidenced by lanes 8 and 18, when the inhibitor and the lysates are combined and then heated, the RT-PCR experiment is a success. Not also that in lanes 5 and 15 (where the inhibitor is heated, but the lysate is not), and in lanes 7 and 17 (where the inhibitor and lysate are heated separately) the RT-PCR experiment fails (indicating that heating the inhibitor in the absence of the lysate "kills" the inhibitor). Surprisingly, however, when the inhibitor and the lysates are combined and then heated, as in lanes 8 and 18, the RT-PCR experiment is successful, indicating a synergy that is more than a sum of the separate effects of the inhibitor, the buffer solution, and heat.

Example 2: Protection of mRNA in Quantitative RT-PCR:

The purpose of this Example is to demonstrate that the present invention will protect mRNA when rat-derived placental RNase inhibitor is used and when human-derived placental RNase inhibitor is used in quantitative RT-PCR experiments wherein rat liver RNases are purposefully added to the reaction.

Materials:

Rat Liver Lysate: 0.5 mg/ml in nanopure water (Sigma Pt # L-1380, Lt # 108F8185)

Luciferase mRNA: 0.1 mg/ml in nanopure water (Promega Pt# L456A, Lt # 14937403)

Kanamycin mRNA: 0.005 mg/ml in nanopure water (Promega Pt # C138A, Lt # 15423602)

"RNasin Plus"-brand inhibitor: 40 units/µl (Promega Pt # N261, Lt # 165682)

Recombinant "RNasin"-brand Inhibitor: 40 units/µl (Promega Pt # N251, Lt # 152734)

AccessQuick™ RT-PCR System (Promega Pt # A1703 Lt # 158304)

Experimental:

The following reactions were assembled without the addition of mRNA:

Table 3:

Rat RNase Inhibitor (Fig. 2):

Reaction #	Luc RNA	Kan RNA	Rat Liver Lysate	Rat RNasin	Nanopure
1	-	-	2.5 µl	20 µl	4. 5 µl
2	2.5 µl	2 µl	-	-	23.0 µl
3	2.5 µl	2 µl	-	20 µl	2.5 µl
4	2.5 µl	2 µl	-	20 µl	2.5 µl
5	2.5 µl	2 µl	-	20 µl	2.5 µl
6	2.5 ul	2 ul	2.5 ul	-	20.0 ul
7	2.5 ul	2 ul	2.5 ul	-	20.0 ul
8	2.5 ul	2 ul	2.5 ul	-	20.0 ul
9	2.5 ul	2 ul	2.5 ul	20 ul	-
10	2.5 ul	2 ul	2.5 ul	20 ul	-
11	2.5 ul	2 ul	2.5 ul	20 ul	-

Table 4:

Human RNase Inhibitor (Fig. 3):

Reaction #	Luc RNA	Kan RNA	Rat Liver Lysate	Human RNasin	Nanopure
1	-	-	2.5 µl	20 µl	4. 5 µl
2	2.5 µl	2 µl	-	-	23.0 µl
3	2.5 µl	2 µl	-	20 µl	2.5 µl
4	2.5 µl	2 µl	-	20 µl	2.5 µl
5	2.5 µl	2 µl	-	20 µl	2.5 µl
6	2.5 µl	2 µl	2.5 µl	-	20.0 µl
7	2.5 µl	2 µl	2.5 µl	-	20.0 µl
8	2.5 µl	2 µl	2.5 µl	-	20.0 µl
9	2.5 µl	2 µl	2.5 µl	20 µl	-
10	2.5 µl	2 µl	2.5 µl	20 µl	-
11	2.5 µl	2 µl	2.5 µl	20 µl	-

The reactions were incubated for 5 minutes at room temperature.

Luciferace mRNA, 2.5 µl of 0.1 mg/ml, (250 ng total) and 2 µl of 0.005 mg/ml kanamycin mRNA (10 ng) were then added to each reaction.

The reactions were incubated at 37°C for 5 minutes.

An RT-PCR master mix was assembled on ice as follows, using components available from Promega Corp.:

250 µl Access Quick 2x Master Mix
200 µl Nuclease-Free Water
10 µl Luciferase Upstream primer # 20939 (15 µM)
10 µl Luciferase Downstream primer # 20979 (15 µM)
10 µl Kanamycin Upstream primer # 20936 (15 µM)
10 µl Kanamycin Downstream primer # 20937 (15 µM)
10 µl AMV-RT (5 units/µl)

Forty-five (45 µl) of the RT-PCR master mix was dispensed into MicroAmp-brand strip-well tubes on ice. Five (5) µl of each reaction was then added to the master mix. The reactions were placed in the PE 9600 thermocycler and cycled as follows:

48°C	45 minutes	1 cycle
96°C	2 minutes	1 cycle
94°C	15 seconds	
65°C -> 55°C	1 minute	12 cycles
72°C	1.5 minutes	
72°C	5 minutes	1 cycle
4°C	Soak	

Twenty (20) µl of each RT-PCR reaction was then loaded onto a 1% TBE agarose gel with ethidium bromide staining and run for 1 hour at 80V.

Results:

The results are shown in Figs. 2, 3, and 4.

Figs. 2 (rat) and 3 (human) - Lane Nos:

1. 200 bp DNA Step Ladder
2. No template Control
3. No Lysate/ no RNasin - Full product
4. No Lysate - Full product
5. No Lysate - Full product
6. No Lysate - Full product
7. + Lysate/ no RNasin
8. + Lysate / no RNasin
9. + Lysate / no RNasin
10. + Lysate + RNasin
11. + Lysate + RNasin
12. + Lysate + RNasin

Quantitation of the band intensities in Fig. 2 and Fig. 3 was performed using densitometry and the ratio of luciferase product (upper band 1.6 Kb) to kanamycin product

(lower band 1.2 Kb) were determined. The ratios were then averaged over n = 3:

Table 5:

	top	Bottom	ratio	Average	SD
2 (+ control)	213875	429975	0.4974	0.5696	0.0867
3 (+ control)	207031	394050	0.5253		
4 (+ control)	208742	301035	0.6934		
5 (+ control)	205821	365820	0.5626		
6 (lysate)	223445	302907	0.7377	0.7467	0.0584
7 (lysate)	228114	267729	0.8520		
8 (lysate)	236778	305877	0.7740		
9 (RNasin)	239481	368160	0.6504	0.6036	0.0701
10 (RNasin)	242121	462849	0.5231		
11 (RNasin)	245322	384800	0.6375		

Table 6:

	AVG	SD
Control	0.5696	0.0867
Lysate-treated	0.7467	0.0584
Lysate + RNasin	0.6036	0.0701

A two-tailed t-test was then performed assuming unequal variances. The results were as follows:

Table 7:
Lysate / Control
t-Test: Two-Sample Assuming Unequal Variances:

	Lysate	*Control*
Mean	0.7879	0.569675
Variance	0.00341103	0.007516916
Observations	3	4
Hypothesized Mean Difference	0	
Df	5	
t Stat	3.973485473	
P(T<=t) one-tail	0.005299643	
t Critical one-tail	2.015049176	
P(T<=t) two-tail	0.010599285	
t Critical two-tail	2.570577635	

Table 8:
Lysate / RNasin
t-Test: Two-Sample Assuming Unequal Variances:

	Lysate	*RNasin*
Mean	0.7879	0.603666667
Variance	0.00341103	0.004909843
Observations	3	3
Hypothesized Mean Difference	0	
Df	4	
t Stat	3.498197957	
P(T<=t) one-tail	0.012468416	
t Critical one-tail	2.131846486	
P(T<=t) two-tail	0.024936833	
t Critical two-tail	2.776450856	

Table 9:
Control/ RNasin -
t-Test: Two-Sample Assuming Unequal Variances:

	RNasin	*Control*
Mean	0.603666667	0.569675
Variance	0.004909843	0.007516916
Observations	3	4
Hypothesized Mean Difference	0	
Df	5	
t Stat	0.573267997	
P(T<=t) one-tail	0.295640794	
t Critical one-tail	2.015049176	
P(T<=t) two-tail	0.591281587	
t Critical two-tail	2.570577635	

For lysate-treated and Full product control, $p < 0.05$, a significant difference.

For lysates-treated and RNasin-protected, $p < 0.05$, a significant difference.

For control Full-product and RNasin-protected, $p > 0.05$, an insignificant difference.

These results are presented graphically in Fig. 4.

This Example shows that there is a significant difference between the lysate-treated samples and the control samples and between the lysate-treated samples and the RNasin-treated samples. There is no significant difference between the control samples and the RNasin-treated samples. In short, there is no difference in the yield of RT-PCR product obtained in the reactions where the inhibitor is added to lysate, but there is a significant difference in the yield of product when no inhibitor is added to the lysate.

Example 3: Effect of Heating RNase in Presence of RNase Inhibitor:

The Example illustrates the effect of heating the RNase in the presence of RNase inhibitor. The experiment was conducted as follows: Agar was mixed with RNA and a pH indicator, Toluidine Blue-O. Specifically, 1.5% LB agar with 0.2% yeast RNA (pH 7.0) was

mixed with 0.005% Toluidine Blue-O. The yeast RNA was purchased from Boehringer-Mannheim (catalog no. 109-223). Toluidine Blue-O was purchased from Sigma (catalog no. T 3260). The agar was poured in a petri dish and allowed to solidify. RNase degradation of RNA releases the nucleotides, thereby decreasing the local pH. This turns the pH indicator pink.

Three solutions were assembled in duplicate in 0.5 ml microfuge tubes. The compositions were as follows:

Table 10:

Component	RNase Alone	RNase + HR	RNase + RR
Water	80 µl	70 µl	70 µl
RNase A (0.1 mg/ml)*	20 µl	20 µl	20 µl
Human RNasin (40 U/µl)	0 µl	10 µl	0 µl
Rat RNasin (40 U/µl	0 µl	0 µl	10 µl

* RNase A without DTT was prepared in a buffer containing Ribonuclease A (Sigma R4875), 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, and 50% glycerol.

One of the duplicate solutions was heated at 70°C for 5 min, and then allowed to cool to room temperature. The other of the duplicate solutions was kept at room temperature the entire time.

The dish was gridded and wells were cored into the gel for loading the different samples. Samples of these solutions were then placed in the wells cored into the agar plate. The plate was then incubated at 37°C for 30 minutes. As shown in Fig. 5, the top half of the plate comprises samples which were heated, while the bottom half comprises samples which were not heated. The heated samples were, from top to bottom, RNase alone; RNase plus human RNase inhibitor; and RNase plus recombinant RNase inhibitor (rat-derived). The non-heated samples are in the same order. From left to right, the lanes show the samples were added in volumes of 2 µl, 2 µl, 5 µl, 5 µl, 10 µl, and 10 µl, respectively.

The results of the experiment show that for both the heated and unheated rows containing the RNase alone, there is a dark halo indicating degradation of RNA. For the rows containing RNase and human RNase inhibitor and rat-derived RNase inhibitor, there is no halo, indicating that there is no degradation of RNA. For the rows containing RNase and human RNase inhibitor or rat-derived RNase inhibitor that were not heated-treated, there is a weak halo around all the cores, indicating that even for non-heat-treated samples, the protection of RNA by RNase inhibitor is not complete. In contrast, there is complete inhibition for the heat-treated samples even at high volumes of added RNase.

Example 4: RNA Degradation By RNase in Presence of Inhibitor, Heated & Non-Heated:

This Example was performed to examine the breakdown of RNA by RNase in the presence of RNase inhibitor and buffer with and without heating. The experiment was performed by preparing two identical agar plates in which the agar was mixed with RNA and a pH indicator.

Five solutions were assembled in duplicate in 0.5ml microfuge tubes. The compositions were:

Table 11:

Component	RNase Alone*	RNase+RNasin	RNase+SB**	RNase+Buffer B***	No RNase (control)
Water	80 µl	70 µl	70 µl	70 µl	100 µl
RNase A*	20 µl	20 µl	20 µl	20 µl	0 µl
RNasin Plus (40 U/µl)	0 µl	10 µl	0 µl	0 µl	0 µl
Storage Buffer**	0 µl	0 µl	10 µl	0 µl	0 µl
Buffer B***	0 µl	0 µl	0 µl	10 µl	0 µl

*RNase A = RNase A (Sigma R4875) was prepared in storage buffer without DTT
** Storage Buffer = 20 mM HEPES-KOH (pH 7.6 at 4°C), 50 mM KCl, 8 mM DTT, 50% (v/v) glycerol
*** Buffer B = 60 mM Tris-Cl, pH 7.5 (at 37°C), 60 mM $MgCl_2$, 500 mM NaCl, 10 mM DTT

One set of the duplicates was heated at 70°C for 5 min, cooled to 4°C, and then allowed to come to room temperature. The other set of duplicates was kept at room temperature the entire time. Samples, 10 µl each, of these solutions were then placed into the wells in the agar plates. The results of this experiment, as illustrated in the schematic in Fig 6.

The plates were loaded identically, with the exception that the plate on the left was loaded with samples incubated at room temperature, while the plate on the right was loaded with samples that were heated to 70°C. The plates were loaded, top to bottom: RNase alone; RNase + "RNasin" RNase inhibitor in Promega Storage Buffer; RNase +storage buffer; RNase + Promega Buffer B. The plates were then incubated at 37°C for 30 minutes. The results of the experiment, shown in Fig. 6, indicate that, for the unheated samples, inhibition of RNase occurs in the presence of the inhibitor only. For the heated samples, inhibition occurs only in the presence of the inhibitor and the storage buffer. These results indicate that for protection of RNA at both room temperature and increased temperatures, inhibitor and buffer must be added while the mix is being prepared at room temperature.

Example 5: Inhibition of Wheat Germ RNases with Rat RNasin:

The purpose of this Example is to determine whether pre-heated rat RNasin is an effective inhibitor of the RNases present in wheat germ extract.

Materials:

Wheat Germ Extract (Promega Pt# L481A, Lt# 12204104)

RNasin Plus: 40 units/µl (Promega Pt# N261, Lt# 165682)

Luciferase mRNA: 1 mg/ml (Promega Pt# L456A, Lt # 14937403)

AccessQuick™ RT-PCR System (Promega Pt# A1703, Lt# 158304)

Experimental:

The following reactions were assembled without addition of luciferase mRNA:

reaction #	Luc mRNA	Nanopure	Wheat Germ Extract	Rat RNasin
1	–	30 µl	–	–
2	1 µl (1 µg)	29 µl	–	–
3	1 µl (1 µg)	9 µl	–	20 µl
4	1 µl (1 µg)	–	1 µl	20 µl
5	1 µl (1 µg)	–	1 µl	20 µl
6	1 µl (1 µg)	9 µl	1 µl	10 µl
7	1 µl (1 µg)	14 µl	1 µl	5 µl

Reaction Nos. 1 through 4 were kept at room temperature. Reaction Nos. 5 through 7 were heated at 70°C for 15 minutes and then allowed to cool to room temperature.

One (1) µl (1 µg) of luciferase mRNA was then added to the reactions as indicated.

The reactions were then incubated at 37°C for 60 minutes.

An RT-PCR master mix was assembled on ice as follows, using components available from Promega Corp.:

250 µl Access Quick 2x Master Mix
220 µl Nuclease Free Water
10 µl Luciferase Upstream primer, Promega Pt. # 20247 (15 µM)
10 µl Luciferase Downstream primer, Promega Pt. # 20818 (15 µM)
10 µl AMV-RT (5 units/µl)

Forty-five (45) µl of the RT-PCR master mix was dispensed into "MicroAmp"-brand strip-well tubes on ice. Five (5) µl of each reaction was then added to the master mix. The reactions were placed in the PE 9600 thermocycler and cycled as follows:

48°C	45 minutes	1 cycle
96°C	2 minutes	1 cycle
94°C	15 seconds	
65°C -> 55°C	1 minute	20 cycles
72°C	1.5 minutes	

72°C	5 minutes	1 cycle
4°C	Soak	

Fifteen (15) µl of each RT-PCR reaction was then loaded onto a 1% TBE agarose gel with ethidium bromide staining and run for 1 hour at 80V.

The results are shown in Fig. 7 (WGE = wheat germ extract):

Lane # 1 - 200 b.p. DNA Step Ladder
2 - No template
3 - Full Product
4 - RNasin Only/ No WGE
5 - WGE + RNasin @ RT
6 - WGE + RNasin @ 70°C (20 µl)
7 - WGE + RNasin @ 70°C (10 µl)
8 - WGE + RNasin @ 70°C (5 µl)

This Example demonstrates that heat-treated rat RNasin is inhibiting some of the RNases present in the wheat germ extract, although the inhibition is not complete. See lane 5 of Fig. 7 and compare to lanes 6, 7, and 8.

Example 6: More Inhibition of Wheat Germ RNases with Rat RNasin:

The purpose of this Example, like that of Example 4, was to determine whether pre-heated rat RNasin is an effective inhibitor of the RNases present in wheat germ extract. Slightly different buffers were used in this Example, including a buffer with and without added DTT (to assess the effects of DTT on the reactions).

Materials:

Wheat Germ Extract (Promega Pt# L481A Lt# 12204104)

RNasin Plus: 40 units/µl (Promega Pt# N261 Lt# 165682)

Luciferase mRNA: 1 mg/ml (Promega Pt# L456A Lt # 14937403)

AccessQuick™ RT-PCR System (Promega Pt# A1703 Lt# 158304)

RNasin Storage Buffer (Promega Pt # BN251 Lt# 147681)

RNasin Storage Buffer plus DTT:

20 mM HEPES-KOH, pH 7.6

50 mM KCl

8 mM DTT

50% glycerol

Experimental:

The following reactions were assembled without addition of luciferase mRNA:

Table 12:

Reaction #	Luc mRNA	Nanopure	Wheat Germ Extract	Rat RNasin	Storage buffer +DTT	Storage buffer - DTT
1	–	30 µl	–	–	–	–
2	1 µl (1 µg)	29 µl	–	–	–	–
3	1 µl (1 µg)	9 µl	–	20 µl	–	–
4	1 µl (1 µg)	28 µl	1 µl	–	–	–
5	1 µl (1 µg)	28 µl	1 µl	–	–	–
6	1 µl (1 µg)	8 µl	1 µl	20 µl	–	–
7	1 µl (1 µg)	8 µl	1 µl	20 µl	–	–
8	1 µl (1 µg)	18 µl	1 µl	10 µl	–	–
9	1 µl (1 µg)	23 µl	1 µl	5 µl	–	–
10	1 µl (1 µg)	8 µl	1 µl	–	20 µl	–
11	1 µl (1 µg)	8 µl	1 µl	–	20 µl	–
12	1 µl (1 µg)	8 µl	1 µl	–	–	20 µl
13	1 µl (1 µg)	8 µl	1 µl	–	–	20 µl

Reaction Nos. 1 through 4, 6, 10, and 12 were kept at room temperature. Reaction Nos. 5 ,7, 8, 9, 11, 13, and 15 were heated at 70°C for 15 minutes and then allowed to cool to room temperature.

One (1) µl (1 µg) of luciferase mRNA was then added to the reactions as indicated.

The reactions were then incubated at 37°C for 60 minutes.

An RT-PCR master mix was assembled on ice as follows:

250 µl Access Quick 2x Master Mix
220 µl Nuclease Free Water
10 µl Luciferase Upstream primer, Promega Pt. # 20247 (15 µM)
10 µl Luciferase Downstream primer Promega Pt. # 20818 (15 µM)
10 µl AMV-RT (5 units/µl)

Forty-five (45) µl of the RT-PCR master mix was dispensed into "MicroAmp"-brand strip-well tubes on ice. Five (5) µl of each reaction was then added to the master mix. The reactions were placed in the PE 9600 thermocycler and cycled as follows:

48°C	45 minutes	1 cycle
96°C	2 minutes	1 cycle
94°C	15 seconds	
65°C -> 55°C	1 minute	20 cycles
72°C	1.5 minutes	
72°C	5 minutes	1 cycle
4°C	Soak	

Fifteen (15) µl of each RT-PCR reaction was then loaded onto a 1% TBE agarose gel with ethidium bromide staining and run for 1 hour at 80V.

The results are shown in Fig. 8.

Lane # 1 - 200 b.p. DNA Step Ladder
2 - No template
3 - Full Product
4 - RNasin Only/ No WGE
5 - WGE RT Only / No RNasin
6 - WGE 70°C Only / No RNasin
7 - WGE RT + RNasin RT
8 - WGE 70°C + RNasin 70°C (20 µl)
9 - WGE 70°C + RNasin 70°C (10 µl)
10 - WGE 70°C + RNasin 70°C (5 µl)

11 - WGE RT + Storage Buffer w/ DTT RT
12 - WGE 70°C + Storage Buffer w/ DTT 70°C

13 - WGE 70°C + Storage Buffer no DTT 70°C
14 - WGE RT + Storage Buffer w/ DTT RT

NB: Reaction Nos. 12 and 13, in lanes 13 and 14, were accidentally inverted upon loading the gel.

As in Example 5, this Example shows that the present invention is capable of inhibiting the wheat germ extract RNases, but not completely. Specifically, compare the amount of product obtained in lane 7 vs. lanes 8 through 10. Also, an interesting observation from lanes 11 through 14: Storage Buffer with or without DTT is capable of providing some protection as long as it is heated. It appears as if all factors contribute in some fashion to the synergistic inhibitory effect seen by the combination of rat RNasin, Storage Buffer, DTT, and heat.

Example 7: RNase Inhibition Without DTT:

In this Example, inhibition of RNase I, an RNase from *E. coli*, (Promega Cat. #M4261), was inhibited by: (1) RNasin Plus-brand RNase inhibitor (Promega Cat. #N261, in storage buffer with 8 mM DTT); and (2) RNasin-brand RNase inhibitor in storage buffer without DTT, following incubation at elevated temperature. The RNasin Plus-brand inhibitor without DTT was purified and stored in buffers that never contained reducing agents, particularly DTT. This inhibitor was then incubated with RNase I in the absence of DTT and other reducing agents. The RNase I was surprisingly inhibited by the solution lacking DTT and any other reducing agent. The fact that RNase I was able to be inhibited by the RNasin Plus-brand inhibitor under such conditions proves that DTT is not absolutely required for RNasin-type inhibitors to inhibit an RNase from *E. coli*.

In addition, solutions of RNase A (Sigma Cat. #R4875) and RNase B (ICN Biomedicals, Cat. #101084) containing RNasin Plus-brand inhibitor were constructed and heated to various temperatures and then tested for RNase activity. In these reactions, no RNase activity was seen upon heating of the RNase/RNase inhibitor solutions. In short, RNase inactivation was shown to be independent of temperature (at the temperatures tested).

These results demonstrate that under the stated conditions, RNasin Plus-type inhibitors, with or without DTT, can completely inhibit these RNases (RNase I, RNase A, RNase B). Moreover, these results show that inhibition is not dependent upon the presence of DTT.

Solutions of RNase I were obtained from Promega Corporation (Cat. #M4261). RNasin Plus-brand inhibitor was also obtained from Promega (Cat. #N261, with 8mm DTT) in storage buffer. Solutions of RNase A (Sigma Cat. #R4875) and RNase B (ICN Biomedicals, Cat. #101084) were purchased, and prepared in storage buffer without DTT, at 100 ng/µl. RNasin Plus-brand inhibitor without DTT was purified by RNase A affinity resin according to the method of Blackburn (1979) *J. Bio. Chem.* 254(24):12484-12487, except without DTT or any other reducing agents in any of the solutions used during purification.

Seven replicate solutions were produced according to Table 13 (adding water to 100 µl):

Table 13:

Solution	Volume	RNase A	RNase B	RNase I	RNasin Plus with DTT	RNasin Plus w/o DTT
A	100 µl	0	0	20 U	0	0
B	100 µl	0	0	20 U	400 U	0
C	100 µl	0	0	20 U	0	400 U
D	100 µl	1 µg	0	0	0	0
E	100 µl	1 µg	0	0	400 U	0
F	100 µl	1 µg	0	0	0	400 U
G	100 µl	0	1 µg	0	0	0
H	100 µl	0	1 µg	0	400 U	0
I	100 µl	0	1 µg	0	0	400 U

One set of each of these tubes was heated for 1 minute using heating temperatures of: 50°C, 60°C, 65°C, 70°C, 80°C, 90°C and 99.9°C. The solutions were then cooled to 4°C and 8 µl from each sample were transferred to wells in an RNase detection plate as described in the earlier Examples. The plate was then incubated at 37°C as described in earlier, and then

read for RNase activity. In Table 14 below, the RNase inhibition was estimated from the size of the zone of RNase activity detected on the RNase detection plate with the 0% inhibition value taken as that seen in the solution incubated at that temperature but in the absence of RNase inhibitor.

Table 14:

Incubation temperature	**% Inhibition Of RNase**								
	A	B	C	D	E	F	G	H	I
50°C	0%	20%	20%	0%	100%	100%	0%	100%	100%
60°C	0%	90%	90%	0%	100%	100%	0%	100%	100%
65°C	0%	100%	100%	0%	100%	100%	0%	100%	100%
70°C	0%	100%	100%	0%	100%	100%	0%	100%	100%
80°C	0%	100%	100%	0%	100%	100%	0%	100%	100%
90°C	0%	100%	100%	0%	100%	100%	0%	100%	100%
99.9°C	0%	100%	100%	0%	100%	100%	0%	100%	100%

In a similar experiment, the amount of RNasin Plus-brand inhibitor without DTT was increased 10-fold (10X). Under these conditions, RNase 1 was inhibited by 100% at 58°C.

It is understood that the invention is not confined to the particular construction and arrangement of parts herein illustrated and described, but embraces such modified forms thereof as come within the scope of the following claims.